PTEN DEFICIENCY IN INTESTINAL EPITHELIAL CELLS INHIBITS AUTOPHAGY BY SUPPRESSING YKT6 EXPRESSION

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ABSTRACT

Phosphatase and tensin homolog (*PTEN*) has long been considered to be a tumor-suppressing gene. Therefore, when *PTEN* is inhibited, it causes tumorigenesis. However, emerging evidence suggests that *PTEN* deficiency alone does not cause tumorigenesis in the intestine. Thus, the crucial role of *PTEN* in the intestine remains elusive. Autophagy is a pathway of cellular waste clearance in the intestine by degrading intracellular waste, such as misfolded proteins. Therefore, autophagy is thought of as a survival mechanism and plays a key role in preventing countless diseases; autophagy inhibition ultimately results in cell death.

As preliminary data, it was found that a loss of the *PTEN* gene suppressed the expression of YKT6 in the intestinal epithelial cells (DLD-1). YKT6 is an R-soluble NSF attachment receptor (SNARE) protein that mediates the fusion between autophagosomes and lysosomes. While various mechanisms take place as a function of autophagy, proper lysosome function is crucial to regulate the process. Because autophagosome-lysosome fusion is required for matured autophagy, impaired YKT6 expression inhibits autophagy. Therefore, it is hypothesized that *PTEN* deficiency inhibits autophagy maturation through YKT6 suppression, leading to cell death. To test this hypothesis, the expression of YKT6 was examined in both control and *PTEN*-knockdown (KD) intestinal epithelial cells. By elucidating the role of YKT6, we hope to further the understanding of chronic inflammatory bowel diseases (IBD) and colorectal cancer.

INTRODUCTION

Along with increasing research into oncogenes and tumor-suppressing genes, the different forms of cell death have also been extensively studied. These types of cell death are primarily divided into two types - those caused by physical or chemical injury, such as necrosis and pyroptosis, or those initiated by the cell itself, such as apoptosis and autophagy. In particular, autophagy plays a vital role in maintaining cellular homeostasis. As a cellular degradation pathway found in all life forms, autophagy is critical to all organisms as a survival mechanism.[1,2] By breaking down senescent and abnormal components of the cell in lysosomes, autophagy can protect the cell from cell death.[3] Because the entirety of autophagy is hugely complex, countless factors contribute to its regulation. From its mechanism of action to its different interactions with cellular compounds, autophagy regulation is affected by many proteins and enzymes.[4,5]

To further understand the mechanism behind autophagy control, tumor-suppressing genes like *PTEN* have been studied. As a gene that suppresses tumor growth, *PTEN* functions to prevent cell growth; therefore, when *PTEN* is inhibited, cells will continue to grow, eventually leading to cancer formation.[6,7] Previous studies have shown that the *PTEN* gene can be an essential regulator of autophagy through the PI3K-Akt-mTOR pathway, indicating that the *PTEN* gene and autophagy are closely related.[6]

Recent research has indicated that there may be a link between the *PTEN* gene, the YKT6 protein, and autophagy. When *PTEN* is deficient in the cell, decreased YKT6 protein levels have been found, and as a result, the formation of autophagosomes also decreases.[8,9] In this study, we hypothesize a possible pyroptotic mechanism of action in connection with *PTEN* and YKT6.

RATIONALE

Gaps in Current Knowledge:

While prior research has demonstrated that *PTEN* deficiency suppresses autophagy, the connection between *PTEN* and YKT6 is yet to be extensively studied. Preliminary research has found that a knockdown (removal) of the *PTEN* gene causes a decreased expression of YKT6 in intestinal epithelial lines (DLD-1). However, DLD-1 represents just one intestinal epithelial cell line - more research is needed with various types of intestinal epithelial cell lines to confirm the hypothesis that *PTEN* deficiency inhibits autophagy in the intestine.

Rationale:

Cancer is currently one of the most researched topics in the 21st century as it is one of the leading causes of death worldwide with no cure. Studies have shown that cancer may result from various elements ranging from genetics to environmental factors, so determining the cause of the cancer is critical to effective treatment for a patient.

The short-term goal of the research is to study the expression of autophagy-regulating genes in *PTEN*-deficient intestinal epithelial cells. In connection with the short-term goal, a long-term goal is to understand the mechanism *PTEN* uses to control autophagy in intestinal epithelial cells. Not only can this research be used to further the study of cancer, but it can be utilized to study different conditions such as inflammatory bowel disorder (IBD).

MATERIALS AND METHODS

Creation of *PTEN* KD Cells with shRNA:

In order to fulfill the short-term goal of this project, we utilized the HT-29 and DLD-1 cell lines, which are two different intestinal epithelial adenocarcinoma lines. HT-29 cells were stably transfected with the human *PTEN* shRNA construct, as previously carried out in a prior study in the laboratory.[10] Several individual clones were obtained, and successful knockdown of *PTEN* expression (PTEN-KD) was confirmed via Western blotting with PTEN protein. Control cells were stably transfected with scrambled shRNA construct, followed by isolation of individual clones.

Replicates:

Each experiment and analytical procedure were performed in triplicates.

Cell Culture and *PTEN* Transfection:

First, we cultivated the HT-29 cells in a controlled environment to ensure no other factors affected the results. By growing the cells in a culture, we could manipulate only the factor of interest, in our case, the effect of *PTEN* deficiency. While culturing the cells, we practiced the basic skills required to maintain and preserve cell cultures. To begin, we prepared Gibco Dulbecco's Modified Eagle Medium (DMEM) for the controls with 1% penicillin-streptomycin (pen-strep) and glutamate, with 10% fetal bovine serum (FBS). Using the created DMEM, we prepared 0.1% puromycin media for the *PTEN* KD cells. After preparing the media for the cells, we incubated both control and KD cells at 37°C until they were ready for protein collection. We continually changed the media and utilized aseptic techniques to prevent cell contamination. While waiting for the cells to adhere to the plate and become confluent, we maintained a working stock of both control and *PTEN* KO cells. Once the cells reached high enough

confluency, we performed cell counting using our lab's automated cell counter (Luna Countess II) and prepared the cells for protein extraction.

We then transfected the cells with either the shPTEN or scrambled construct using the SuperFect transfection reagent (QIAGEN) following the protocol provided by the manufacturer. Then, we selected transfected cells using the puromycin (2 μg/mL) media. Then, we utilized stably transfected cells for the experiment.

Western Blotting:

Using a radioimmunoprecipitation assay (RIPA) buffer with a phosphatase inhibitor cocktail, cells were lysed and mechanically scrapped with a cell tissue scraper, and protein was extracted.

Finally, we conducted a western blot with the collected proteins to analyze YKT6 expression in both of our DLD-1 cell lines. After creating a 10% gel and loading ~40 μg of protein in each well, we ran the gel through electrophoresis at 70 V for 17 hours so that the protein bands could separate over time. Afterward, we transferred proteins from the gel to a polyvinylidene difluoride (PVDF) membrane using a wet western blot transfer at 70 V and 500 mA for 4 hours. Once the proteins from the gel had been transferred to the membrane, we blocked them in a 5% nonfat milk solution with a tris-buffered saline (TBS) for 1 hour to prevent further unwanted reactions between the PVDF membrane and proteins. Then, we exposed the membrane to the YKT6 primary antibody (1:500) overnight at 4°C (Table 1). Next, we incubated the sample with goat anti-rat IgG Alex Fluor 488 secondary antibody (1:1000) for 2 hours, followed by TBS-T washes. Finally, the membranes were analyzed with the ChemiDoc Touch Imaging System in the Department of Biology at Oakland University.

Antibody	Source	Concentration	Manufacturer
YKT6 Primary	Rabbit	1:500	Abcam
PTEN Primary	Mouse	1:1000	Santa Cruz Biotechnology
IgG Alexa Fluor 488 Secondary	Goat	1:1000	Abcam

Table 1: **Antibodies utilized in Western Blot.** Various antibodies from multiple sources and varying concentrations were utilized to detect protein presence in HT-29 and DLD-1 intestinal epithelial cells. Primary antibodies were used to detect respective proteins on the PVDF membrane, and secondary antibodies were used to detect the presence of primary antibodies through the ChemiDoc Touch Imaging System.

LPS Cell Transfection:

Using techniques learned during the maintenance of cell cultures, HT-29 cells were grown until they were confluent enough for transfection with lipopolysaccharide (LPS), an endotoxin associated with cellular integrity and inflammatory responses. LPS transfection was completed following the protocol provided by the manufacturer of the Lipofectamine 2000 DNA Transfection Reagent, Thermo Fisher Scientific.

After determining if the cells grew to a 70-90% confluency with a binocular compound microscope on a Petri dish (90 mm diameter), Lipofectamine reagent was diluted in DMEM media (1:15) [mixture A] and LPS in DMEM media (1:1400) [mixture B] to incubate for 5 minutes. Following the incubation period, mixtures A and B were combined to treat HT-29 cells (4:1) with LPS over 26 and 48 hours. The cells were later imaged with the Olympus VS120

Virtual Imaging System regulated by the Department of Biology at Oakland University. After several trials, the ratios of mixture A, B, and the final solution were determined experimentally.

RESULTS

PTEN KD and DLD-1 Cells:

To verify the transfection and knockdown (KD) of *PTEN* in DLD-1 cells, a western blot was performed to test *PTEN* levels using the *PTEN* primary antibody (Fig. 1A).[11] The lack of a signal at 50 kilodaltons (kDa) in the DLD-1 *PTEN*-KD column, as opposed to the presence of a signal at 50 kDa in the DLD-1-*PTEN*-WT column, confirmed that the *PTEN* gene was successfully knocked down in *PTEN*-WT cells. Using the same western blot, we observed decreased levels of YKT6 in DLD-1-*PTEN*-KD cells compared to DLD-1-*PTEN*-WT cells. The blot also shows similar levels of β-actin in types of DLD-1 cells, indicating that similar levels of protein were loaded into each well during the analysis.

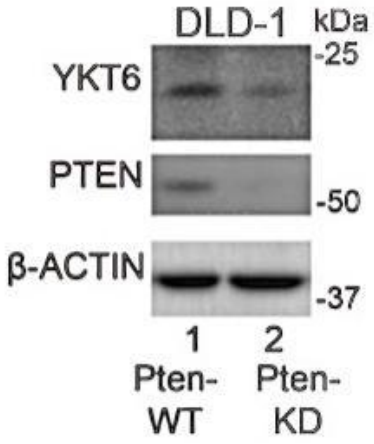

Figure 1A: ***PTEN* deficiency downregulates the level of YKT6 in DLD-1.** DLD-1 *PTEN*-WT and *PTEN*-KD cells were subjected to western blotting to evaluate YKT6, *PTEN*, and β-actin levels. From 11/2021.

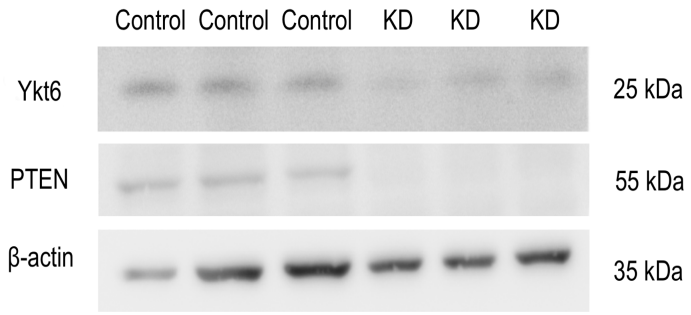

Figure 1B: ***PTEN* deficiency downregulates the level of YKT6 in DLD-1.** DLD-1 *PTEN*-WT and *PTEN*-KD cells were subjected to western blotting to evaluate YKT6, *PTEN*, and β-actin levels. From 12/2020.

Before our western blot skills developed over time, results from figure 1B indicate that the experimenter's skills are critical to obtaining accurate results. Figure 1B displays that *PTEN* deficiency downregulates YKT6 levels, like Fig. 1A. However, there is little contrast, with the axes of data being presented in non-chronological order and triplicates cluttering the results.

PTEN KD and HT-29 Cells:

To verify the transfection and knockdown (KD) of *PTEN* in HT-29 cells, a western blot was performed to test *PTEN* levels using the *PTEN* primary antibody (Fig. 2). The lack of a signal at 25 kilodaltons (kDa) in the HT-29 *PTEN*-KD column, as opposed to the presence of a signal at 25 kDa in the HT-29-*PTEN*-WT column, confirmed that the *PTEN* gene was successfully knocked out in *PTEN*-KD cells. Using the same western blot, we could see decreased levels of YKT6 in HT-29-*PTEN*-KD cells compared to HT-29-*PTEN*-WT cells. The blot also shows similar levels of β-actin in types of DLD-1 cells, indicating that similar levels of protein were loaded into each well during the analysis.

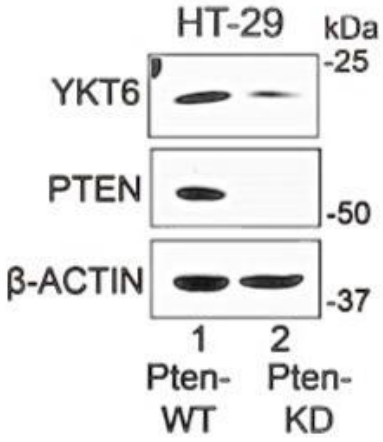

Figure 2: ***PTEN* deficiency downregulates the level of YKT6 in HT-29.** HT-29 *PTEN*-WT and *PTEN*-KD cells were subjected to western blotting to evaluate YKT6, *PTEN*, and β-actin levels.

LPS Transfection for 26 Hours - 10X Magnification:

Noting the connection between autophagy and pyroptosis in mediating cell death, we investigated the induction of cell death with lipopolysaccharide (LPS), a glycolipid and endotoxin known for its ability to induce strong inflammatory responses at 26 hours of incubation (Fig. 3).[12] Because cell death results in a compromised cell membrane, cell death could be approximated by measuring cell death. This was accomplished with a trypan blue dye assay, where the number of cells that had trypan blue dye entered the membrane, the greater the cell death. The results indicate increased cell death in HT-29 cells incubated with LPS and the transfection reagent rather than the transfection reagent alone.

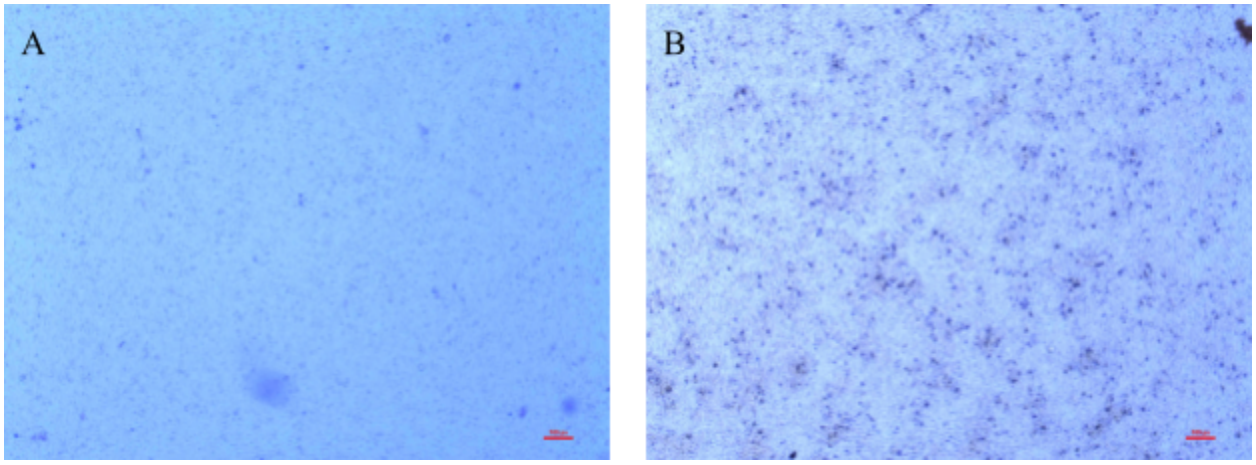

Figure 3: HT-29 Cell Transfection with LPS increases cell death. (A) HT-29 cells were incubated for 26 hours with only the transfection agent, displaying minimal cell death. (B) HT-29 cells were incubated for 26 hours with the transfection agent and LPS, displaying increased cell death compared to (A).

LPS Transfection for 48 Hours - 10X Magnification:

In continuation of our study into LPS-mediated pyroptosis, cell death was investigated at 48 hours of incubation (Fig. 4). Like Fig. 3, because cell death results in a compromised cell membrane, cell death could be approximated with the number of cells that had trypan blue dye enter. The results indicate increased cell death in HT-29 cells incubated with LPS and the transfection reagent rather than the transfection reagent alone. Interestingly, HT-29 cells transfected with LPS and the transfection reagent for 48 hours exhibited increased cell death compared to 26 hours, indicating that the more extended incubation period led to increased cell death.

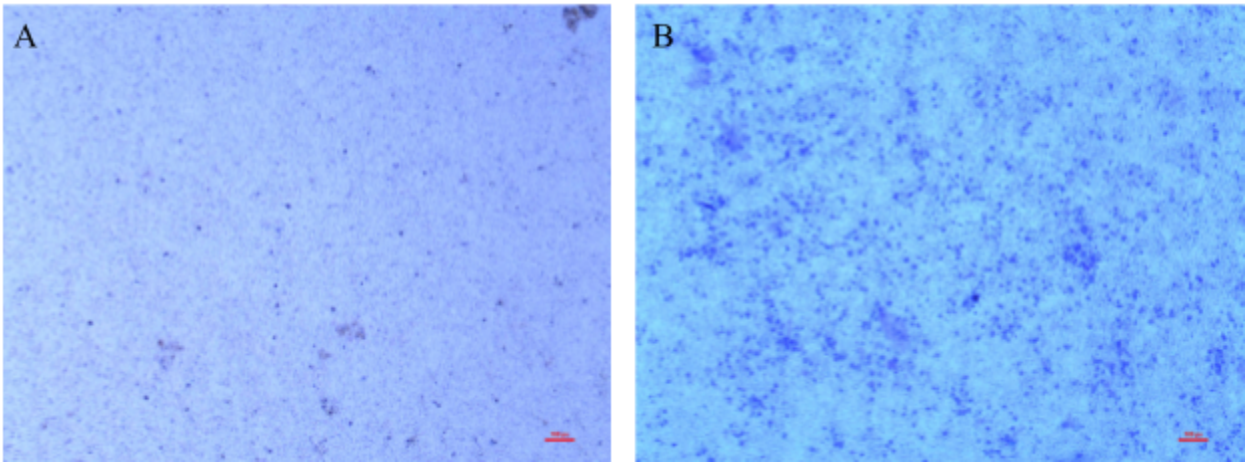

Figure 4: HT-29 Cell Transfection with LPS increases cell death. (A) HT-29 cells were incubated for 48 hours with only the transfection agent, showing minimal cell death. (B) HT-29 cells were incubated for 48 hours with the transfection agent and LPS, displaying high cell death rates.

DISCUSSION

PTEN, YKT6, and Autophagy:

Phosphatase and tensin homolog (*PTEN*) tumor-suppressing gene that leads to the production of a lipid phosphate that cleaves phosphatidylinositol (3,4,5)-triphosphate (PIP3) to phosphatidylinositol (4,5)-biphosphate (PIP2) via phosphoinositide 3-kinase (PI3K).[5] Thus, *PTEN*-deficient conditions increase the intracellular level of PIP3 because PIP3 cannot be cleaved to PIP2. This accumulation of PIP3 in *PTEN* deficiency induces the activity of protein kinase B (Akt), which activates the mammalian target of rapamycin (mTOR).[13] mTOR acts as an autophagy inhibitor, one of two major complexes that regulate the process of autophagy. mTOR acts by phosphorylating the ATG13 kinase, preventing it from binding with ATG1 kinase to form the autophagosome.[14] Because autophagosome and lysosome fusion is required to form the autolysosome, which initiates autophagy in the cell, *PTEN* deficiency ultimately decreases the activation of autophagy, promoting cell death. Therefore, a knockdown (KD) of the *PTEN* gene results in cell death.

The possibility of autophagy inhibition leading to cell death was considered in studying the different mechanisms of how *PTEN* inhibition leads to cell death. Autophagy is an essential cell mechanism regulated by the lysosome organelle. Autophagy degrades self-antigens such as misfolded protein or damaged self-proteins.[1,2] Autophagy is also responsible for cellular waste clearance to maintain homeostasis; therefore, it plays a key role in preventing autoimmune diseases and infections.[1] Accordingly, the inhibition of autophagy leads to apoptotic and non-apoptotic cell death.[4,5]

For a cell to undergo autophagy, however, lysosomes must fuse with closed autophagosomes to achieve degradation.[8] Because lysosomes contain digestive enzymes to

regulate autophagy, if autophagosome-lysosome fusion were to be inhibited, lysosomes would not be able to function appropriately; autophagy would be inhibited.[8] YKT6 is an autophagosomal SNARE protein and mediates this autophagosome-lysosome fusion - therefore, YKT6 inhibition inhibits autophagosome-lysosome fusion and, ultimately, autophagy (Fig. 5).[8,9]

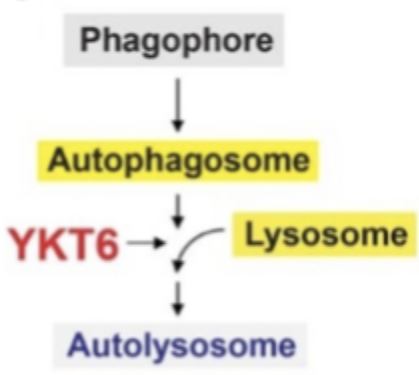

Figure 5: YKT6 is pivotal mediator for the autophagosome-lysosom e fusion.

In this study, it was concluded that *PTEN* deficiency leads to markedly decreased levels in YKT6, ultimately leading to the suppression of autophagy in intestinal epithelial cells (Fig. 1, Fig. 2). DLD-1 and HT-29 cell lines derived from human colorectal cells function as intestinal epithelial cells due to being isolated from the colon.

PTEN Regulation of YKT6:

While the exact mechanism of *PTEN* regulating YKT6 levels has not been identified, possible mechanisms of the connection between the tumor-suppressing gene and the critical protein have been proposed. In particular, syntaxin interaction and microRNA-135b (miR-135b), an onco-miRNA, dysregulation has been raised.

Syntaxin 3 (STX3), a protein involved in vesicle transport and exocytosis, has been found to decrease *PTEN* levels to activate *PTEN*-PI3K-Akt-mTOR signaling in prior studies done in breast cancer cells.[15] STX3 has been shown to bind to *PTEN*, leading to its ubiquitination and degradation, promoting the activity of PI3K to increase levels of PIP3 from PIP2.[15] PIP3 accumulation promotes autophagy, ultimately indicating that STX3 is capable of inducing autophagy. While STX3 does not regulate YKT6, it is a part of the same SNARE complex as

syntaxin 5 (STX5), along with GS28 and GS15.[16] Not much is known about the interaction between STX3 and STX5 - however, the two may be connected as they are part of the same family of membrane proteins and carry out similar functions in exocytosis.

Autophagy and Pyroptosis:

Pyroptosis is another type of cell death caused by injury, mediated by inflammatory caspases and interleukins. Recent research has found that autophagy decreases pyroptosis through the ATGs associated with its own mechanism; decreased ATG levels leads to the overactivation of pyroptosis and cytokine release associated with pyroptosis.[17] Specifically, studies have indicated that removal of ATG7 levels (ATG7 KD) in mice led to increased inflammasome activity in the macrophage, accompanied by increased IL-1β and IL-18 levels associated with pyroptosis.[16]

Specifically, prior studies have found that Immunity-Related GTPase Family M (IRGM) interacts with the ATG8 family in autophagy - IRGM induces LPS-induced caspase activation, which has been hypothesized to be linked with pyroptosis. With LPS being a significant component of the outer membrane of Gram-negative bacteria and contributing to the structural integrity of the cell, it is crucial in the innate immune responses regarding host defense.[18] Therefore, LPS deficiency causes cell death due to structural instability, but LPS overabundance can also cause cell death due to pro-inflammatory-LPS (P-LPS).[15]

In this study, it was observed that transfecting HT-29 cells with LPS resulted in increased cell death compared to non-transfected cells (Fig. 3, Fig. 4). These results were further differentiated when cells were transfected for 48 hours compared to 26 hours. Therefore, this evidence strongly supports that *PTEN* deficiency decreases the expression of YKT6, leading to

decreased autophagy and increased cell death, likely via apoptosis. However, further research is required to confirm or reject this hypothesis, particularly in connection with autophagy.

CONCLUSION

This experiment was performed to determine the role of *PTEN* in regulating YKT6 levels in intestinal epithelial cells. YKT6, a SNARE protein, functions as an anchor for the autophagosome-lysosome fusion necessary for autophagy. This experiment supports the hypothesis that *PTEN* plays a critical role in autophagy through YKT6 regulation and, in turn, tumorigenesis. When *PTEN* was knocked out, YKT6 levels decreased significantly, indicating that *PTEN* deficiency results in autophagy inhibition, ultimately leading to increased cell death.

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